PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference NdP/00319	**FOR FURTHER ACTION** See Form PCT/IPEA/416	
International application No. PCT/IB2005/000795	International filing date *(day/month/year)* 23.03.2005	Priority date *(day/month/year)* 26.03.2004
International Patent Classification (IPC) or national classification and IPC INV. G01N21/64 G01N33/52 C07K14/435		
Applicant RIZZUTO, Rosario et al.		

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 7 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☒ *sent to the applicant and to the International Bureau)* a total of 8 sheets, as follows:
      ☒ sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in electronic form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).

4. This report contains indications relating to the following items:

- ☒ Box No. I Basis of the report
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

Date of submission of the demand	Date of completion of this report
03.04.2006	20.06.2006
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Vogt, T Telephone No. +49 89 2399-8477

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/IB2005/000795

Box No. I Basis of the report

1. With regard to the **language**, this report is based on

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of:
   - ☐ international search (under Rules 12.3(a) and 23.1(b))
   - ☐ publication of the international application (under Rule 12.4(a))
   - ☐ international preliminary examination (under Rules 55.2(a) and/or 55.3(a))

2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

Description, Pages

1-47 as originally filed

Claims, Numbers

1-43 received on 10.04.2006 with letter of 03.04.2006

Drawings, Sheets

1/8-8/8 as originally filed

☒ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☐ The amendments have resulted in the cancellation of:
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

* *If item 4 applies, some or all of these sheets may be marked "superseded."*

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/IB2005/000795

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☒ the entire international application,

   ☐ claims Nos.

   because:

   ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination *(specify)*:

   ☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*.

   ☒ no international search report has been established for the said claims Nos. 1-43

   ☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

      ☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Preliminary Examining Authority in a form and manner acceptable to it.

      ☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Preliminary Examining Authority in a form and manner acceptable to it.

      ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rules 13*ter*.1(a) or (b) and 13*ter*.2.

   ☐ a meaningful opinion could not be formed without the tables related to the sequence listings; the applicant did not, within the prescribed time limit, furnish such tables in electronic form complying with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions, and such tables were not available to the International Preliminary Examining Authority in a form and manner acceptable to it.

   ☐ the tables related to the nucleotide and/or amino acid sequence listing, if in electronic form only, do not comply with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions.

   ☐ See separate sheet for further details

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-43
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-43
Industrial applicability (IA)	Yes:	Claims	1-43
	No:	Claims	

2. Citations and explanations (Rule 70.7):

see separate sheet

Supplemental Box relating to Sequence Listing

Continuation of Box I, item 2:

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this report was established on the basis of:

   a. type of material:

   ☒ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☒ on paper

   ☒ in electronic form

   c. time of filing/furnishing:

   ☐ contained in the international application as filed

   ☐ filed together with the international application in electronic form

   ☒ furnished subsequently to this Authority for the purposes of search and/or examination

   ☐ received by this Authority as an amendment* on

2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

* *If item 4 in Box No. I applies, the listing and/or table(s) related thereto, which form part of the basis of the report, may be marked "superseded."*

III No opinion.

The subject matter of the claims **as originally filed** was found to lack unity of invention and was divided by the international search authority into the following five inventions:

- Invention 1: Claims 1-5 and 44-46 (all partly).
  A method for the screening of molecules that can modify intracellular parameters characterised in that the second messenger itself causes a proportional variation in the intracellular Ca2+ concentration.

- Invention 2: Claims 6-21, 47-50 and 1-5, 44-46 (partly)
  A method for the screening of molecules that can modify intracellular parameters characterised in that a chimeric recombinant Ca2+ sensitive photo-protein probe is used comprising an cellular effector and Ca2+ sensitive photo-protein.

- Invention 3: Claims 22-30, and 1-5 (partly).
  A method for the screening of molecules that can modify intracellular parameters characterised in that a recombinant receptor is used that comprises an intracellular portion that is capable of inducing a variation in the intracellular Ca2+ concentration.

- Invention 4: Claims 34-42, and 31-33, 43 (partly).
  A Ca2+ sensitive photo-protein probe characterised in that it comprise an effector portion and a Ca2+ sensitive photo-protein portion.

- Invention 5: Claims 31-33 and 43 (all partly)
  A Ca2+ sensitive photo-protein probe characterised in that it comprises a Ca2+ sensitive photo-protein portion and a signal sequence.

The applicant was invited to pay additional search fees, but decided neither to pay such fees nor to file a protest against the lack of unity objection raised by the ISA. The international search report was therefore restricted to the subject matter of the first invention mentioned above.

The subject matter of the present independent claims 1, 5, 20, 28, and 33 is however

directed to embodiments falling within inventions 2-5 as identified above.

It follows that the presently claimed subject matter was not the subject of the international search report and can therefore not be the subject of the international preliminary examination report (Rule 66.1(e) PCT).

Moreover it is noted that the subject matter of invention 2, as identified above, must be further subdivided into separate sub-inventions, comprising at least:

- Invention 2(a):

  Subject matter of invention 2, characterised in that the cellular effector portion is coupled to translocation.

- Invention 2(b):

  Subject matter of invention 2, characterised in that the cellular effector portion is coupled to the activation or inactivation state of a protein.

This subdivision is in line with the argumentation of the applicant, provided with the letter of 03.04.2006, with regard to the number of inventions present in the set of claims submitted with said letter.

The requirements of Art. 34(2)(b) PCT were not assessed for the presently claimed subject matter.

CLAIMS

1. Screening method of molecules capable of generating the alteration of a target intracellular parameter, said alteration being the variation in the concentration of a second messenger that is a cyclic nucleotide, said alteration being converted into a proportional variation in the intracellular concentration of the Ca2+ ion, detected by means of a Ca2+-sensitive recombinant aequorin probe, comprising the following phases:

a) construction of an expression vector containing the fusion protein sequence encoding said probe, said sequence being characterized in that it comprises the Ca2+-sensitive recombinant aequorin encoding sequence, condensed together with at least one signal sequence;

b) transfection of at least one of a mammalian cell line with said vector containing the Ca2+-sensitive recombinant aequorin probe, said cell line being previously engineered so as to express an heterologous chimeric receptor being characterized in that it has the intracellular portion of a receptor coupled with variations in the concentration of calcium and the extra-cellular portion of a receptor coupled with the production of cyclic nucleotides;

c) activation of said Ca2+-sensitive aequorin probe by the addition of a prosthetic group to the cellular line expressing said recombinant protein probe;

d) administration of the molecule to be tested to the cellular line expressing said recombinant protein probe;

e) detection of the emission of photons on the part of the Ca2+-sensitive aequorin probe expressed in the cellular line and evaluate the amount of activation or inhibition exerted by the tested molecule, on the basis of a ratio between the cps value obtained and the

maximum value of cps registered under conditions of maximum stimulation of the cellular line.

2. Screening method according to claim 1, wherein said prosthetic group is celentherazine.

3. Screening method according to claims 1 or 2, wherein said cyclic nucleotide is c-AMP.

4. Screening method according to anyone of the claims 1 to 3, wherein said signal sequence directs the Ca2+-sensitive recombinant aequorin probe to a cellular compartment, said probe being the fusion protein mt-aequorin (mt-AEQ).

5. Screening method of molecules capable of generating the alteration of a target intracellular parameter, said alteration being the translocation from cytoplasm to the membrane of a cellular effector, said translocation being correlated to the different intracellular concentration of the Ca2+ ion between cytoplasm and submembrane area, detected by means of a Ca2+-sensitive recombinant aequorin probe, comprising the following phases:

a) construction of an expression vector containing the fusion protein sequence encoding said probe, said sequence being characterized in that it comprises sequences encoding at least one Ca2+-sensitive recombinant aequorin encoding sequence, condensed together with at least one cellular effector and/or a signal sequence;

b) transfection of at least one mammalian cell line with said vector containing the Ca2+-sensitive recombinant protein probe;

c) activation of said Ca2+-sensitive photoprotein by the addition of a prosthetic group to the cellular line expressing said recombinant protein probe;

d) administration of the molecule to be tested to the cellular line expressing said recombinant protein probe;

e) detection of the emission of photons on the part of the Ca2+-sensitive photo-protein expressed in the cellular line and evaluate the amount of activation or inhibition exerted by the tested molecule, on the basis of a ratio between the cps value obtained and the maximum value of cps registered under conditions of maximum stimulation of the cellular line.

6. Screening method according to claim 5, wherein said prosthetic group is celentherazine.

7. Screening method according to claims 5 or 6, wherein said cellular effector is a regulating protein.

8. Screening method according to claim 7, wherein said regulating protein is selected from the group which comprises protein-kinase, phosphatase, adenylate cyclase, proteins that link plasmatic membrane receptors, proteins that interact with plasmatic membrane channels, proteins that interact with plasmatic membrane lipids.

9. Screening method according to anyone of the claims 5 to 8, wherein said signal sequence directs the Ca2+-sensitive recombinant aequorin probe to a cellular compartment.

10. Screening method according to anyone of the claims 5 to 9, wherein said probe is a fusion protein selected from the group that consists of PKC-aequorin (PKC-AEQ) and shc-aequorin (shc-AEQ).

11. Screening method according to claim 10, wherein the PKC-aequorin is selected from the group comprising PKC beta-aequorin, PKC delta-aequorin, PKC epsilon-aequorin, PKC zeta-aequorin, PKC gamma-aequorin, PKC alpha-aequorin, PKC-lambda-aequorin, PKC theta-aequorin, PKC eta-aequorin.

12. Screening method according to claim 10, wherein the shc-aequorin is selected from the group con-

sisting of p66shc-aequorin, p46shc-aequorin, p52shc-aequorin.

13. Screening method according to anyone of the claims 5 to 12, wherein the expression vector of phase a) is a eukaryotic vector.

14. Screening method according to anyone of the claims 5 to 13, wherein said at least one mammal cellular line of phase b) is previously engineered so as to express a heterologous native or chimeric protein.

15. Screening method according to claim 14, wherein said heterologous protein is selected from the group which consists of a receptor, an enzyme, a ionic channel and a cellular effector.

16. Screening method according to claim 14, wherein said chimeric protein is a chimeric receptor.

17. Screening method according to claim 15, wherein said ionic channel is selected from the group which comprises voltage-dependent Ca2+ channels and Ca2+ channel receptors.

18. Screening method according to claim 15, wherein said cellular effector is a regulating protein selected from the group which comprises protein-kinase, phosphatase, adenylate cyclase, proteins that links plasmatic membrane receptors, proteins that interacts with plasmatic membrane channels, proteins that interacts with plasmatic membrane lipids.

19. Screening method according to claim 15, wherein said cellular effector is a cell membrane receptor selected from the group which comprises receptors coupled with G proteins, receptors with an enzymatic activity, channel receptors.

20. A Ca2+-sensitive recombinant fusion protein probe, characterized in that it comprises the sequence encoding at least one Ca2+-sensitive recombinant aequorin encoding sequence, condensed together with at least one cellular effector and/or a signal sequence,

said cellular effector being a regulating protein selected from a protein-kinase and a protein that links plasmatic membrane receptors.

21. Probe according to claim 20, wherein said protein-kinase is a protein kinase C (PKC).

22. Probe according to claim 21, wherein said PKC-aequorin is selected from the group which comprises PKC beta-aequorin (PCK beta: rif. M13975), PKC delta-aequorin (PCK delta: rif. M18330), PKC epsilon-aequorin (PCK epsilon: rif. AF028009), PKC zeta-aequorin (PCK zeta: rif. M18332), PKC gamma-aequorin, PKC alpha-aequorin (PCK alfa: rif. M13973), PKC-lambda-aequorin, PKC theta-aequorin (PCK theta: rif. L07032), PKC eta-aequorin.

23. Probe according to claim 20, wherein said protein that links plasmatic membrane receptors is an adaptor protein.

24. Probe according to claim 23, wherein said adaptor protein belongs to the shc family.

25. Probe according to claim 24, wherein the protein is selected from the group comprising p46shc, p52shc and p66shc.

26. Probe according to claim 20, wherein said cell membrane receptor is selected from the group which comprises receptors coupled with G proteins, receptors with an enzymatic activity, channel receptors.

27. Probe according to claims 20 to 26, wherein said signal sequence directs the Ca2+-sensitive photo-protein, preferably aequorin, towards a cellular compartment.

28. Use of the Ca2+-sensitive recombinant fusion protein probe as defined in claims 20 to 27, for the screening of molecules capable of generating the alteration of an intracellular parameter, said alteration being the translocation from cytoplasm to the membrane of a cellular effector, said translocation being correlated to

the different intracellular concentration of the Ca2+ ion between cytoplasm and submembrane area.

29. Use according to claim 28, wherein said cellular effector is selected from the group which consists of ionic channel, regulating protein, cell membrane receptor.

30. Use according to claim 29, wherein said ionic channel is selected from the group which comprises voltage-dependent Ca2+ channels and Ca2+ channel receptors.

31. Use according to claim 29, wherein said regulating protein is selected from the group which comprises protein-kinase, phosphatase, adenylate cyclase, proteins that links plasmatic membrane receptors, proteins that interacts with plasmatic membrane channels, proteins that interacts with plasmatic membrane lipids.

32. Use according to claim 29, wherein said cell membrane receptor is selected from the group which comprises receptors coupled with G proteins, receptors with an enzymatic activity, channel receptors.

33. Screening method of molecules capable of generating the alteration of a target intracellular parameter, said alteration being the activation/inactivation of a cellular effector that is a protein kinase acting on Ca2+ ionic channels, said alteration being converted into a proportional variation in the intracellular concentration of the Ca2+ ion, detected by means of a Ca2+-sensitive recombinant aequorin probe, comprising the following phases:

a) construction of an expression vector containing the fusion protein sequence encoding said probe, said sequence being characterized in that it comprises the Ca2+-sensitive recombinant aequorin encoding sequence, condensed together with at least one signal sequence;

b) transfection of at least one of a mammalian cell line with said vector containing the Ca2+-sensitive recombinant aequorin probe;

c) activation of said Ca2+-sensitive aequorin probe by the addition of a prosthetic group to the cellular line expressing said recombinant protein probe;

d) administration of the molecule to be tested to the cellular line expressing said recombinant protein probe;

e) detection of the emission of photons on the part of the Ca2+-sensitive aequorin probe expressed in the cellular line and evaluate the amount of activation or inhibition exerted by the tested molecule, on the basis of a ratio between the cps value obtained and the maximum value of cps registered under conditions of maximum stimulation of the cellular line.

34. Screening method according to claim 33, wherein said prosthetic group is celentherazine.

35. Screening method according to claim 33 or 34, wherein said cellular effector is PKC.

36. Screening method according to anyone of the claims 33 to 35, wherein said Ca2+ ionic channel is selected from the group which comprises voltage dependent Ca2+ channels and Ca2+ channel-receptors.

37. Screening method according to claim 36, wherein said Ca2+ channels are L type Ca2+ channels.

38. Screening method according to anyone of the claims 33 to 37, wherein said signal sequence directs the Ca2+-sensitive recombinant aequorin probe to a cellular compartment.

39. Screening method according to anyone of the claims 33 to 38, said probe being a fusion protein selected from the group which comprises SNAP aequorin (SNAP-AEQ), mt-aequorin (mt-AEQ) and cytosol aequorin (cyt-AEQ).

40. Screening method according to anyone of the claims 33 to 39, wherein said at least one mammal cellular line of phase b) is previously engineered so as to express a heterologous native or chimeric protein.

41. Screening method according to claim 40, wherein said heterologous protein is selected from the group which consists of ionic channels.

42. Screening method according to claim 41, wherein said ionic channel is selected from the group which comprises voltage-dependent Ca2+ channels and Ca2+ channel receptors.

43. Screening method according to claim 42, wherein said Ca2+ channels are L type Ca2+ channels.